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NITROGUANIDYL-LUTROPIN, A DERIVATIVE WHICH INHIBITS THE STIMULATION OF OVARIAN ADENYLATE CYCLASE BY LUTROPIN

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1. Introduction

The high reactivity of the ϵ-NH_2 group of lysine residues offers the possibility of introducing very different groups in the sidechain of this amino acid. These groups can alter different characteristics: e.g., length of the chain, charge, distance between the charge and the peptide backbone. When the positive charge of the ϵ-NH_2 groups of lysine in luteinizing hormone is suppressed, the biological activity of this hormone is abolished [1,2]. However, if the positive charge is maintained, various chemical modifications of the ϵ-NH_2 group can lead to gradual modification of the biological properties of the molecule ranging from the preservation of the total biological activity (e.g., with the methylated derivative) [1] to a combination of agonist and antagonist properties (e.g., with some guanidinated derivatives) [3].

With the nitroguanidyl group, the positive charge is suppressed and the size of the introduced group rather increased in comparison to the methylated or guanidinated derivatives. This paper describes the physico-chemical and biological properties of the nitroguanidyl derivative of LH, the first well-defined antagonist obtained by chemical modification of the protein moiety in the gonadotropins.

2. Materials and methods

Ovine LH was prepared in a highly purified state (biological potency: 2 X LH-NIH, S11) as in [4].

Abbreviations: LH, luteinizing hormone (lutropin); FSH, follicle-stimulating hormone (follitropin); G, guanidyl; NG, nitroguanidyl

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Lutropin subunits were obtained by the countercurrent distribution method in [5]. Ovine follicle-stimulating hormone was kindly provided by the National Pituitary Agency, Baltimore (MA) (NIAMDD-OFSH-13 = 15, NIHFSH S1). Cholera enterotoxin (27.8 lb/g Lowry protein) was purchased from Schwarz Mann (Orangeburg, NY) and prostaglandin E_2 from Sigma. 1-Nitroguanyl-3,5-dimethyl pyrazol (Eastman Organic Chemicals) was used for nitroguanidylation, as in [6]. The degree of nitroguanidylation of amino acid residues was determined by amino acid analysis using a Technicon analyzer [6].

Sucrose gradient sedimentation was done as in [7]. Ovine LH was used as reference protein. Iffa Credo rats, 22 days old, were killed and the ovaries rapidly excised and minced. Using a loose-fitting glass homogenizer the ovaries were homogenized in 5 mM Tris–HCl buffer (pH 7.4) containing 10 mM $MgCl_2$ and 100 mM sucrose. Ten up-and-down strokes were given with a Teflon rod. The homogenate (100 mg wet tissue/ml buffer) was then filtered through a layer of cheese cloth. The incubation medium for adenylate cyclase assay contained 33 mM Tris–HCl buffer (pH 7.4), 3 mM $MgSO_4$, 2.5 mM NaCl, 0.1 mM GTP, 0.1 mM ATP, bovine serum albumin 0.2%, 11 mM phosphoenol pyruvate, pyruvate kinase 80 μg/ml, 8 mM theophylline and various amounts of hormone or derivatives. The reaction was started by adding the homogenate (700 μg protein). It was allowed to proceed for 10 min. at 37°C and stopped by addition of trichloroacetic acid to give 5% final conc. The aliqots were used for determination of cAMP accumulation after centrifugation and extraction with diethyl ether [8].

The amount of cAMP was established as in [9]. Radioactivity determinations were done using a Tricarb Packard liquid scintillation spectrometer.

3. Results

3.1. *Preparation and characterization of the nitroguanidyl derivative*

Lutropin was treated by nitroguanyldimethylpyrazole at room temperature in the dark for 24 h (10 mg protein, 40 mg reagent, 1 ml borate buffer, pH 10.3). The solution was then dialysed; first against a solution containing pyridine (1%) and NH_4HCO_3 (1%) and thereafter against a pyridine solution (1%). It was lyophylized and submitted to gel filtration on Biogel P-30 and Biogel P-100 columns. Amino acid analysis showed that ~90% of the lysine residues were modified under these conditions. The preparation of the NG subunits was achieved in a similar manner (87% of lysine residues were modified). The elution volumes in the Biogel P-100 column (fig. 1) indicated an increase of the Stokes radius (r_S) of the proteins after nitroguanidylation. NG-LH has r_S ~37 Å, (native hormone, 30 Å), and the NG subunits r_S ~30 Å, (isolated subunit, 24 Å).

In sucrose gradient sedimentation experiments, the values found for the ratio $s_{NG\text{-}LH}/s_{LH}$ (s, sedimentation coefficient; NG-LH, nitroguanidyl LH) was 1.7. corresponding to a ratio of $M_{NG\text{-}LH}/M_{LH}$ (M, mol. wt) of ~2.2. It may be concluded that in aqueous solution 2 molecules of NG-LH associate. This may be due to the more hydrophobic character of the NG-group compared with the NH_2 group.

3.2. *Biological characterization*

Fig. 2 shows the cyclic AMP accumulation obtained by incubation of juvenile rat ovarian homogenates in the presence of either LH or NG-LH. The presence of NG-LH in the medium induces a decrease of the cyclic AMP content of the homogenates under the basal level instead of the accumulation observed in the presence of LH.

Table 1
Inhibition by addition of NG-LH of the LH-induced stimulation of ovarian adenylate cyclase

Treatment	Remaining % stimulation (mean ± SEM)[a]
LH[b]	100
LH[b] + 60 μg NG-LH	66.50 ± 5.45
LH[b] + 120 μg NG-LH	55.25 ± 8.76
LH[b] + 180 μg NG-LH	34.75 ± 3.88

[a] Each value represents the mean of 4 determinations
[b] Ovine LH, 80 μg/ml

This inhibitory action of the NG-LH is clearly demonstrated when LH is added to the medium at the same time as NG-LH. As shown in table 1, the adenylate cyclase stimulation induced by a constant amount of LH is decreased by the action of increasing concentration of NG-LH. When the incubation is performed in the presence of a constant amount of NG-LH and variable amounts of native LH (fig. 3), the cyclic AMP accumulation is lower than that obtained in the presence of LH alone, demonstrating once again the inhibitory action of the derivative. However, increasing

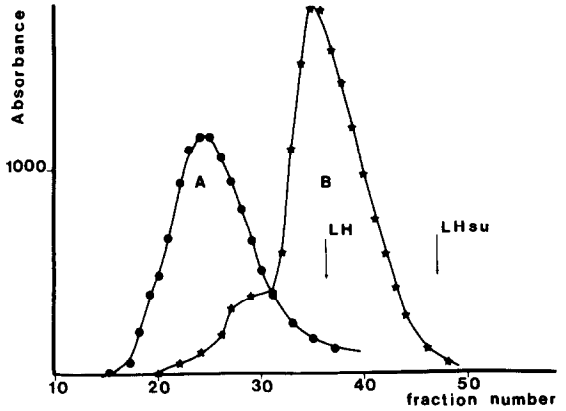

Fig.1. Gel filtration on Biogel P-100 of 10 mg NG-LH (peak A) and 10 μg NG-LH α subunit (peak B). Arrows indicate the elution volume of native LH and of the LH subunit.

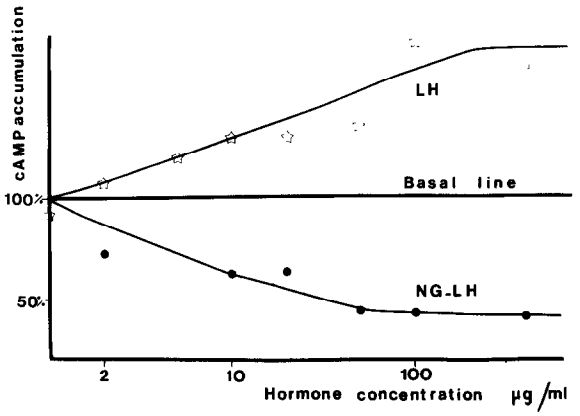

Fig.2. Cyclic AMP accumulation induced in ovarian homogenates from juvenile rat by LH and NG-LH. 100% corresponds to 40 pmol cAMP/mg protein.

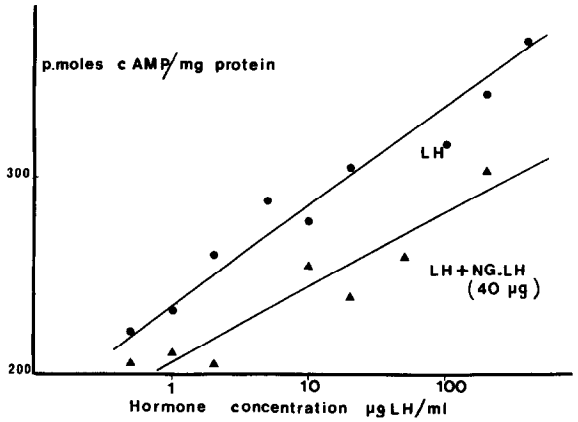

Fig.3. Cyclic AMP accumulation induced in rat ovarian homogenates by increasing amounts of LH in the presence or in the absence of 40 µg NG-LH.

amounts of LH can compensate for the inhibitory effect of NG-LH suggesting a competitive inhibition of the action of the hormone by the derivative.

The specificity of this inhibitory action was investigated by incubating ovarian adenylate cyclase in the presence of different stimulating agents and NG-LH (table 2). No significant inhibitory effect on the stimulating action of azide, fluoride, prostaglandin E_2 or choleragen was observed. NG-LH did not antagonize FSH.

No inhibitory action on LH stimulation was displayed by nitroguanidinated serum albumin. The dependence of the action of NG-LH on its quaternary structure is indicated by fact that neither NG-(LHα) nor NG-(LHβ) (nitroguanidinated subunits) exhibit inhibitory properties on LH action.

Table 2
Effect of NG-LH on the stimulation of ovarian adenylate cyclase induced by different effectors or by FSH[a]

Effector	NG-LH (µg/ml)	Stimulation[b] (%)
Azide 200 mM	120	97.3 ± 6.6
	180	99.8 ± 8.7
	200	99.7 ± 6.7
NaF 10 mM	60	90.3 ± 6.9
	120	100.7 ± 5.8
	180	90.6 ± 4.5
PGE_2, 10 µg/ml	50	99.6 ± 2.7
	100	95.3 ± 9.7
Cholera toxin		
5 µg/ml	150	110.0 ± 6.0
20 µg/ml	150	99.5 ± 6.5
50 µg/ml	150	105.5 ± 8.5
FSH 1 µg	50	104.0 ± 3.0
	100	94.5 ± 1.5
	200	100.0 ± 4.0

[a] The cAMP accumulation induced by the effector without addition of NGLH was considered as 100% stimulation
[b] Mean values and SEM obtained from 3 different expt

4. Discussion

Some guanidylated derivatives can act at the same time as weak agonists and as antagonists [3]. Binding to the receptors and adenylate cyclase stimulation occur, but the steroidogenic effect is very low [3]. In comparison to the guanidyl derivatives, the NG-derivative offers, from a chemical point of view, some new features. The presence of the NO_2 group suppresses the basic character of the guanidyl group. Another difference is the less hydrophilic character of the NG-group. We have seen that as a result of these new features, the agonistic character of the derivative is abolished but its antagonistic properties are maintained and their scope extended to the stimulation of adenylate cyclase by LH.

Our results underlined, on the other hand, the high specificity of nitroguanidyl lutropin in its action on cyclic AMP accumulation. NG-LH is without effect on the adenylate cyclase stimulation induced by fluoride, azide and prostaglandin E_2. The action of FSH on juvenile ovarian adenylate cyclase which is mediated by FSH specific receptors different from those of LH, is unaltered by the presence of NG-LH.

It must also be pointed out that NG-LH is without effect on the adenylate cyclase stimulation induced by the cholera toxin. Recent investigation of hormone-dependent adenylate cyclases indicates indeed that the stimulating action of choleragen and that of hormones are quite distinct and produced by different mechanisms [10,11].

A diminution of the cyclic AMP accumulation under the usual baseline is obtained when the ovarian homogenates are incubated in the presence of NG-LH (fig. 3). Since the action of the derivative is very specific, it seems reasonable to think that endogenous LH (or LH-like molecules) is responsible, at least in part, for the increase in the content of cyclic AMP usually produced during incubation of ovarian homogenates without addition of effectors.

NG-LH can act also as an antagonist of the action of LH on Leydig cells [12], inhibiting as well as the binding of LH to the membrane receptors, the cyclic AMP accumulation and the testosterone production. Some similarities exist therefore between the competitive inhibitory actions of NG- and that of G-LH [3]. However, there also exists a fundamental difference which must be emphasised: the NG-LH–receptor complex is unable to activate the membrane adenylate cyclase from any 'compartment' whereas G-LH activates various gonadal adenylate cyclases [3,8]. NG-LH can act, therefore as an inhibitor of the stimulation of adenylate cyclase by LH whereas G-LH cannot.

NG-LH is the first derivative obtained by chemical modification of the protein moiety of LH which acts exclusively as an antagonist. As could be expected, this action is exhibited only by the LH derivative but not by the derivatives obtained from the pre-separated subunits. By modification of the sugar moiety, some LH derivatives have been prepared which are also able to act as inhibitors of some activities of LH [13–16]. All these inhibitors will afford new possibilities for studying the mechanism of action of LH and for investigating its intervention in the physiology of reproduction.

Acknowledgements

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